JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1996, p. 2850–2852 Vol. 34, No. 11
0095-1137/96/$04.00+0

Diagnosis of *Neospora caninum* and *Toxoplasma gondii* Infection by PCR and DNA Hybridization Immunoassay

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Received 1 July 1996/Returned for modification 12 August 1996/Accepted 27 August 1996

A recently described PCR test for the identification of *Neospora caninum* and *Toxoplasma gondii* has been further developed and optimized in view of its practicability for routine diagnostic application. The *N. caninum*-specific PCR was adapted to the diagnostic operating standard of the *T. gondii*-specific PCR in that the uracil DNA glycosidase system was introduced, which eliminates potential carry-over contaminations of amplified target DNA from previous reactions. Furthermore, both PCR tests were optimized by including a DNA hybridization immunoassay based on the use of the commercially available Gen-eti-k DEIA kit. This assay allowed highly sensitive and specific detection of respective DNA amplification products and thus substantially facilitated the reading and interpretation of the test results.

Neospora caninum and *Toxoplasma gondii* are cyst-forming coccidian parasites of veterinary clinical relevance primarily because of the neurological symptoms they cause. The two parasites are also well known for causing congenital infections associated with abortion and/or severe damage of the fetus. In humans, only *T. gondii* has been demonstrated so far, showing the same range of neuropathological and congenital damage as listed above.

In the last few years, diagnosis of neosporosis and toxoplasmosis was much improved by the development of PCR tests which allow a fast and methodically highly sensitive identification of the parasite through the amplification, and subsequent demonstration, of parasite-specific DNA sequences (1, 3, 6, 10). The present report describes the elaboration of a reliable and specific DNA hybridization immunoassay (DIA) on the basis of a previously descibed method (8), which, in contrast to a conventional DNA analysis by gel electrophoresis, provides unambiguity in the detection of *N. caninum*- and *T. gondii*-specific diagnostic amplification products. This technical achievement may considerably simplify the routine application of PCR procedures in diagnosis of the two coccidian parasites.

For PCR-based diagnosis of *N. caninum*, the Nc5 region (5, 10) was selected as the target sequence for DNA amplification. PCR was performed in a 50-μl mixture containing 5 μl of 10× Gene Amp PCR buffer (Perkin Elmer Cetus, Norwalk, Conn.); 0.2 mM each dATP, dGTP and dCTP; 0.4 mM dUTP (Pharmacia, Dübendorf, Switzerland); 20 pmol each of *N. caninum*-specific primers Np21plus (5′CCCAGTGCGTCCAATCCTG TAAC3′) and Np6plus (5′CTCGCCAGTCAACCTACGTCT TCT3′); 1.25 U of AmpliTaq DNA polymerase (Perkin Elmer Cetus, Rotkreuz, Switzerland); and 0.5 U of uracyl DNA glycosylase (UDG) (GibcoBRL, Basel, Switzerland) (see above). UDG and dUTP (instead of dTTP) were included in the reaction mixture to remove eventual (dUTP containing) carry-over contaminations from previous diagnostic reactions according to a method elaborated by Longo and coworkers (7). For UDG-mediated decontamination prior to PCR, the reaction mixture was initially incubated for 2 min at 50°C. This incubation was followed by a 5-min denaturation of the DNA at 95°C. Subsequently, amplification was done in a thermal cycler (Perkin Elmer Cetus, Rotkreuz, Switzerland) using 40 cycles with denaturation (94°C; 1 min), annealing (63°C; 1 min), and primer extension (74°C; 3.5 min). After the last cycle, a primer extension was continued for 10 min at 74°C, and finally 50 μl of chloroform was added to inactivate UDG. PCR for detection of *T. gondii* was done by the method of Bretagne and coworkers (1) using the 35-fold-repetitive B1 gene (3) as a target for DNA amplification. All reactions were done on 1-μl aliquots of previously heat-denatured (by 5 min of boiling) DNA samples prepared from parasite material as described below. Furthermore, false-negative results, caused by inhibitory compounds in the PCR tests, were excluded by performing a simultaneous positive control reaction on the samples in presence of *N. caninum* or *T. gondii* DNA equivalents from about five parasites (not shown). Amplification products were analyzed by electrophoresis through a 2% agarose gel (9) for the *N. caninum*-specific PCR or a 10% polyacrylamide gel (10% Tris-Borate-EDTA Ready Gels, purchased from Biorad, Glattbrugg, Switzerland) for the *T. gondii*-specific PCR, respectively. In parallel, amplification products were detected by applying the Gen-eti-k DEIA kit (Sorin Biomedica, Saluggia, Italy) essentially according to the instructions from the purchaser. Briefly, streptavidin-coated wells from microtiter plates were sensitized with 50 ng (in 100 μl of phosphate-buffered saline, pH 7.2) of a 5′ biotinylated oligonucleotide capture probe specific for *N. caninum* (5′bio-GGTGAACCGAGGGA GTTGGT3′) or *T. gondii* (5′bio-ACGGTCCGGGTGAAACA ATAGAG3′) DNA amplification products, respectively. Subsequently, 10-μl aliquots of the PCR samples were denaturated by 5 min of boiling and hybridized to the respective probes for 1 h at 45°C. Hybrids between the capture probe and the trapped nucleotide strand from the amplification products were detected by their binding activity to a double-strand-specific mouse monoclonal antibody and by a subsequent colorimetric method based on the use of an anti-mouse immunoglobulin antibody-alkaline phosphatase conjugate (Promega, Madison, Wis.) at a dilution of 1:500.

Figure 1 shows the PCR results visualized either by gel electrophoresis analysis or by the DIA procedure. PCR testing included the amplification of *N. caninum*- and *T. gondii*-specific genomic DNA as well as of the phylogenetically closely

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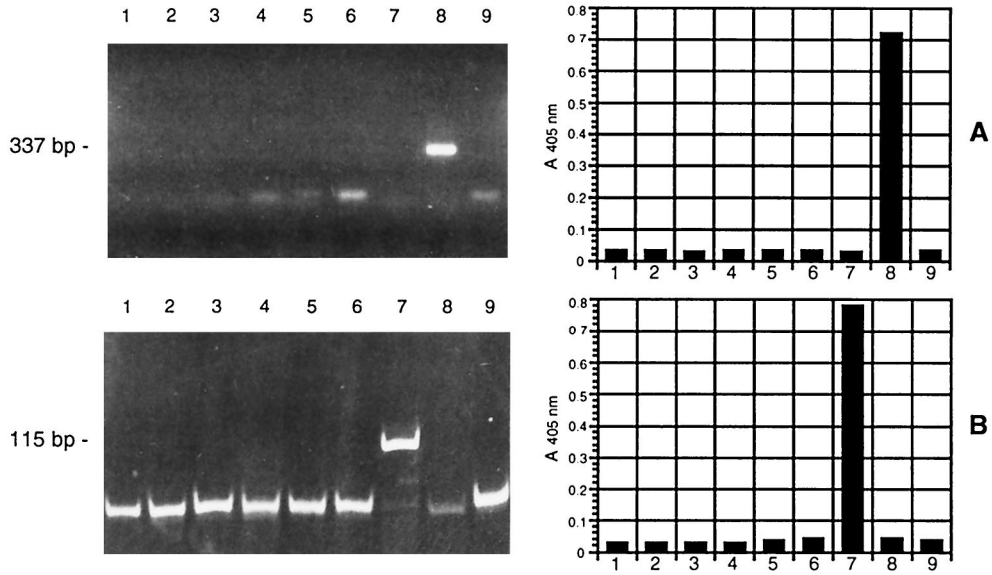

FIG. 1. DIA for specific PCR-based diagnosis of *N. caninum* (A) and *T. gondii* (B). Amplification products from PCRs on 10 ng of *H. hammondi* (lanes 1), *S. tenella* (lanes 2), *S. moulei* (lanes 3), *S. mieschericana* (lanes 4), *S. cruzi* (lanes 5), *S. capracanis* (lanes 6), *T. gondii* (lanes 7), and *N. caninum* (lanes 8) DNA and from a control PCR containing no DNA template (lanes 9) were analyzed by 2% agarose (*N. caninum*-specific PCR) or 10% polyacrylamide (*T. gondii*-specific PCR) gel electrophoresis (left side) and DIA (right side). The diagnostic amplification products from the *N. caninum*-specific (337 bp) and *T. gondii*-specific (115 bp) PCR are indicated on the leftmost side of the gels. The bands in the lower part of the gels are diagnostically and methodically irrelevant.

related organisms *Hammondia hammondi* and *Sarcocystis* spp. DNA preparations were done as described elsewere (10). Regarding *Sarcocystis* and *Hammondia* DNA, all PCRs scored negative in both detection systems, which, on the other hand, allowed specific and thus differential diagnosis between *N. caninum* DNA and *T. gondii* DNA. An evaluation of the methodical sensitivity of the *N. caninum*-specific (Fig. 2A, panel a) and *T. gondii*-specific (Fig. 2B, panel a) PCR tests revealed that the respective reactions with DNA derived from one single parasite provided DNA amplification products clearly detectable either by gel electrophoresis or by DIA. In contrast, the two detection systems exhibited qualitatively different results when an analogous test was performed under diagnostically realistic conditions (Fig. 2A and B, panels b and c). In this case, DNA from progressively reducing numbers of parasite cells were prepared in presence of bovine skeletal muscle and brain tissue (kindly provided by M. Welle, Institute of Animal Pathology, Berne, Switzerland), which both are considered to be materials with potential diagnostic importance (4). Preparation of DNA was performed as follows. Approximately 100 mg of tissue was cut with a sterile scalpel blade into small pieces and was then supplemented with *N. caninum* or *T. gondii* tachyzoites that had been isolated and purified according to the method of Brindley and coworkers (2). This material was resuspended in 500 μl of 100 mM NaCl–10 mM Tris-HCl (pH 7.5)–5 mM EDTA–0.5% sodium dodecyl sulfate–500 μg of proteinase K (Sigma, Buchs, Switzerland) per ml and incubated for 4 h at 37°C. Subsequently, DNA was extracted by incubation of the suspension with 500 μl of buffered phenol (UltraPure; GibcoBRL, Basel, Switzerland) for 1 h at 37°C, followed by double treatments with 500 μl of buffered phenol and 500 μl of chloroform (Merck, Darmstadt, Federal Republic of Germany). DNA in the remaining aqueous phase was precipitated with a double volume of ethanol in presence of 300 mM K-acetate, pH 5.2, and after 10 min of sedimentation at 14,000 × *g*, the resulting pellet was washed in 70% ethanol and resuspended in 100 μl of distilled water. For each PCR, a 1-μl aliquot was used, which corresponded to the equivalent of 1 mg of original tissue. As can be seen in Fig. 2, both, gel electrophoresis- and DIA-based analysis of the *T. gondii*-specific PCR allowed detection of amplification products from muscle and brain tissue sample aliquots containing only one single parasite genome (Fig. 2B, panels b and c). However, this examination (see Fig. 2B, panel c, lane 4), as well as various investigations in our routine diagnosis (not shown), demonstrated that the occasional appearance of extra DNA amplification bands may complicate the interpretation of the results from the gel analysis. In such cases, an unambiguous and reliable result was obtained only by performing the appropriate DIA confirmation test. Regarding the *N. caninum*-specific PCRs, demonstration by gel electrophoresis of amplification products from reactions on experimental muscle and brain material (see Fig. 2A, panels b and c) as well as amplification products from reactions on material sampled for routine diagnosis (not shown) was often substantially hampered by the existence of a background smears at the predicted location of the diagnostic band. In these cases again, a clear-cut discrimination between positive and negative results was much facilitated by applying a respective DIA confirmation test.

In conclusion, our study demonstrated that the reliability of PCR-based diagnosis of both *N. caninum* and *T. gondii* was much improved when using DIA either as the ultimate assay or at least as confirmation test for detection of respective DNA amplification products. Besides its excellent diagnostic operating characteristics, the DIA offers the advantage that it can be applied in the 96-well format of a microtiter plate by basically using the technology of the enzyme-linked immunosorbent assay. The possibility of large-format testing makes the DIA system an excellent tool for extended analyses of diagnostic PCRs which may be important for, e.g., large-scale epidemiological studies such as representative surveys on the prevalence of neosporosis and toxoplasmosis in man and domestic animals.

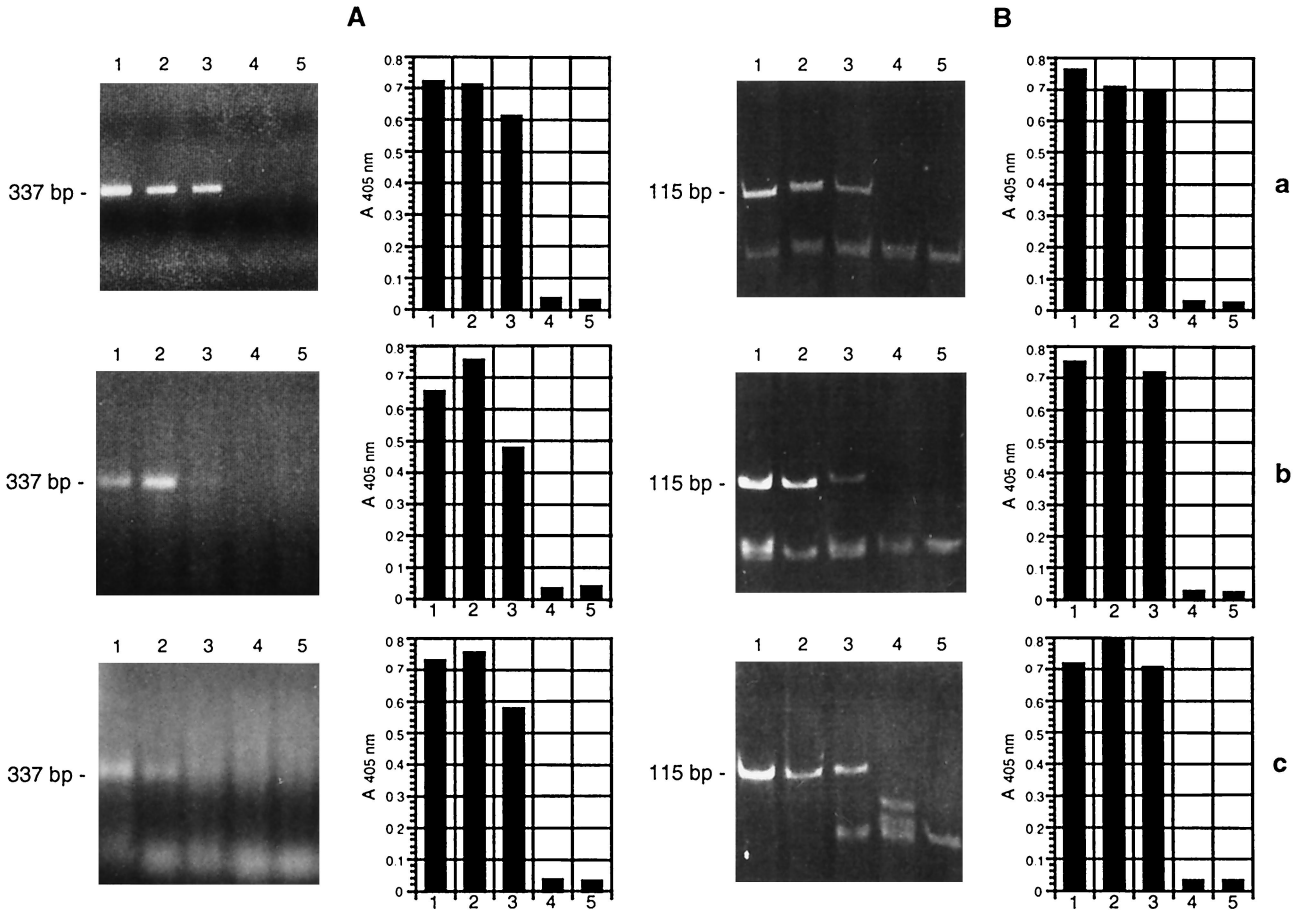

FIG. 2. DIA for sensitive PCR-based diagnosis of *N. caninum* (A) and *T. gondii* (B). Extrapolated on one PCR aliquot, *N. caninum* or *T. gondii* tachyzoites were diluted to 100 (lanes 1), 10 (lanes 2), 1 (lanes 3) 0.1 (lanes 4), and 0 (lanes 5) cells and DNA was prepared for amplification in the absence (panel a) or presence of 1 mg of skeletal muscle (panel b) or brain tissue (panel c). Amplification products were analyzed by 2% agarose (*N. caninum*-specific PCR) or 10% polyacrylamide (*T. gondii*-specific PCR) gel electrophoresis (left side) and DIA (right side). The diagnostic amplification products from the *N. caninum*-specific (337 bp) and *T. gondii*-specific (115 bp) PCR are indicated on the leftmost side of the gels. The bands in the lower part of the gels are diagnostically and methodically irrelevant.

Gen-eti-k DEIA kits were kindly provided by Sorin Biomedica.

This work was supported by the "Swiss Federal Veterinary Office," research grant no. 002.4.3.93.1.

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